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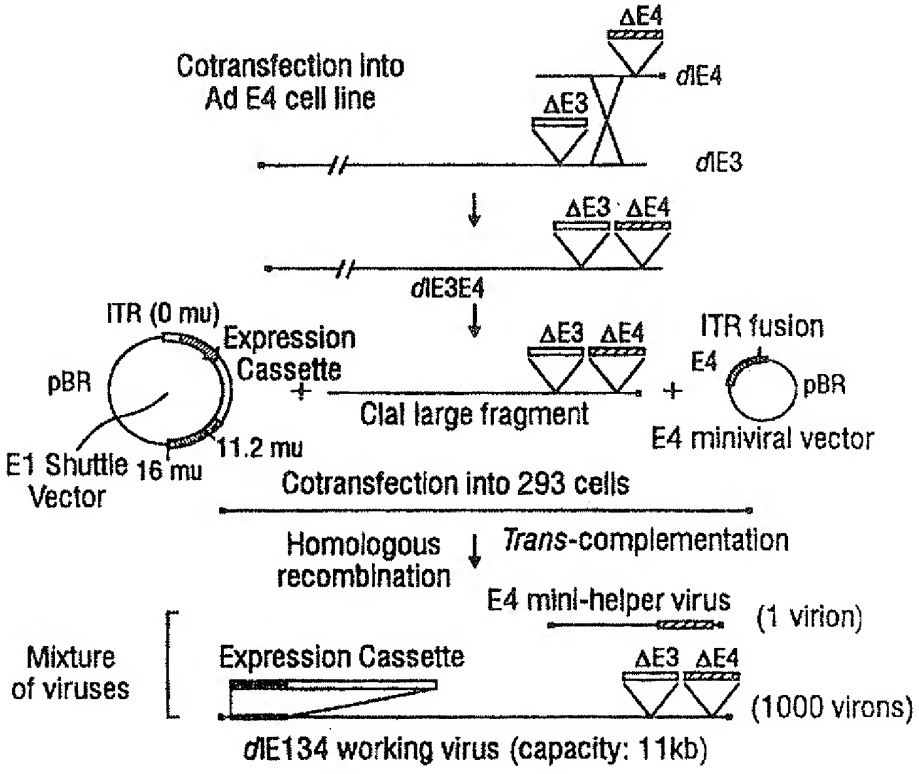
(54) Title: AN ADENOVIRUS HELPER-VIRUS SYSTEM

(57) Abstract

An adenoviral helper viruses system is disclosed that is capable of expressing up to 36 kB of heterologous DNA in a replication defective adenoviral vector. The system comprises adenoviral vector constructs, one or more helper viruses and a helper cell line. The vector construct is capable of being replicated and packaged into a virion particle in the helper cell when coinfected with a helper virus that contains a defective packaging signal. In particular, the helper cell expresses DNA from one or more of the "early" codings regions of the adenovirus 5 genome (Ad5) and one or more helper viruses express DNA from one or more of the "early" coding regions and all of the later coding regions of the Ad5 genome, complementing mutations in the corresponding regions of the vector. Also disclosed are methods of transferring heterologous DNA-containing vectors into mammalian cells.



all adenoviral coding regions. Such vectors can carry about 10, 15, 20, 30 or 35 kB of foreign DNA.

In another embodiment, there is provided an isolated adenovirus vector, wherein said vector comprises an adenoviral inverted terminal repeat and an adenoviral packaging signal and a non-functional, non-immunogenic form of (i) the adenoviral products E1A, E1B and E3; and (ii) at least one of the adenoviral products selected from the group consisting of E2, E4, L1, L2, L3, L4 and L5.

There also is provided an isolated adenoviral helper virus, wherein said virus comprises (i) an adenoviral terminal repeat; (ii) an adenoviral packaging sequence; and (iii) the coding region for at least one of the adenoviral products E1A, E1B, E2, E3, E4, L1, L2, L3, L4 and L5. In another embodiment, there is provided an isolated adenoviral helper virus, wherein said virus comprises (i)an adenoviral terminal repeat; (ii) a mutated adenoviral packaging sequence that is utilized less efficiently than a wild-type adenoviral packaging sequence; and (iii) the coding region for at least one of the adenoviral products E1A, E1B, E2, E3, E4, L1, L2, L3, L4 and L5.

In yet another embodiment, there is provided a method of propagating an adenovirus vector lacking at least part of the coding regions for (a) the adenoviral products E1A, E1B and E3 and (b) at least one of the adenoviral products selected from the group consisting of E2, E4, L1, L2, L3, L4 and L5 comprising the steps of:

(i) providing a cell permissive for growth of an adenovirus defective in the functions provided by adenoviral products of E1A and E1B;

(ii) providing an adenoviral helper virus that complements the absence of the adenoviral product or products as set forth in part (b) above;

(iii) importing said vector and said helper virus into said cell; and

(iv) incubating said cell under conditions that permit replication of said vector.

In still yet another embodiment, there is provided a method of expressing a gene in a mammalian cell comprising the steps of:

(i) providing an adenoviral vector lacking at least part of the coding regions for (a) the adenoviral products E1A, E1B and E3 and (b) at least one of the adenoviral products selected from the group consisting of E2, E4, L1, L2, L3, L4 and L5, wherein the lacking coding regions are replaced by a heterologous DNA encoding said gene;

(ii) propagating said vector under conditions permissive for replication and packaging of said vector in an infectious form;

(iii) isolating propagated vector in an infectious form;

(iv) contacting said infectious form of said vector with said mammalian cell; and

(v) incubating said mammalian cell under conditions such that said foreign gene is expressed.

In a further embodiment, there is provided a method of inhibiting the expression of a gene in a mammalian cell comprising the steps of:

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Gene therapy generally involves three principal elements: therapeutic genes, delivery systems and target cells. One of the urgent technical challenges in gene therapy technology is how to specifically deliver and controllably express the therapeutic genes in target cells *in vivo*. Currently available delivery system are limited in their ability to accomplish these goals (Mulligan, 1993), and there is a great demand for a new system with these capabilities.

The present invention is submitted to represent a significant advance in the genetic engineering of adenoviral vectors and their use in transfer of heterologous DNA into mammalian cells, particularly in the context of gene therapy. This new system will not only substantially increase the gene-delivery capacity of adenoviral vectors, but will also greatly improve their therapeutic potential, since the replacement of the viral genome eliminates the vector-borne cytotoxicity and the possibility of wild-type recombination events that are associated with the current Ad vector systems. Because the helper cell/helper virus system is capable of supporting a wide variety of mutations, the potential use for this system is extensive.

Replication of Ad mutants with deletions in different regions of the viral genome can be supported by helper viruses that provide the deleted gene products in *trans*. One example of this phenomenon involves the case of adenovirus/SV40 hybrid recombinants. Gluzman and Van Doren (1983) identified a recombinant that contained about 3500 base pairs from the left end of the Ad5 viral genome followed by 2.7 copies of the SV40 genome. This structure was repeated in the opposite orientation and, therefore, contained two Ad5 inverted terminal repeats

gene from the adenoviral genome or from another cell, has been introduced. Therefore, recombinant cells are distinguishable from naturally-occurring cells which do not contain a recombinantly-introduced gene. Recombinant cells are thus cells having a gene or genes introduced through "the hand of man."

Replication is determined by contacting a layer of uninfected cells, or cells infected with one or more helper viruses, with virus particles, followed by incubation of the cells. The formation of viral plaques, or cell free areas in the cell layer, is the result of cell lysis caused by the expression of certain viral products. Cell lysis is indicative of viral replication.

**D. Vectors**

Another embodiment of the present invention is an adenovirus vector construct in which at least a portion of the E1 and E3 regions of the virus are deleted, along with at least portion of the E4 and/or E2 regions. In an alternative embodiment, the defects in the E1 and E3 regions may not be deletions but point mutations rendering the "early" gene products inactive or preventing their synthesis entirely. Examples of preferred embodiments provided herein make use of the adenovirus 5 serotype (Ad5) genome. It is understood, however, that other serotypes such as the adenovirus type 2 (Ad2) genome, for example, would also function in the practice of the invention.

Three benefits arise from various forms of adenovirus mutants. Where a mutation simply renders a protein non-functional, the ability of the virus to replicate once administered to a patient is eliminated, thus lessening the chance for pathogenic effects. If the protein mutation also results in the absence of a protein

product, an additional benefit in terms of lower toxicity is realized. Finally, if the adenovirus mutant lacks some or all of the gene segment encoding the protein, the apathogenic and non-toxic phenotype is achieved along with increased capacity to carry foreign genes. Thus, an adenovirus mutant lacking at least a portion of its coding sequence is preferred.

The invention also can be described as an adenovirus vector construct comprising at least about 350 base pairs of the left ITR region of the Ad5 genome, up to about 35 kB of heterologous DNA, and at least about 100 base pairs of the right ITR region of the Ad5 genome. See FIG. 1. Corresponding regions of other serotypes, such as the adenovirus type 2 genome, can be used as well. In its most preferred embodiment, the left and right ITR regions will flank the heterologous DNA and contain said heterologous DNA between them. Any arrangement of the viral and heterologous DNA that permits replication and encapsidation is acceptable, however, and is included as a part of the present invention.

Prior to the present invention, the largest insert that could be contained in the vector was 5.5 kB, inserted in place of the E1 and E3 regions and including the additional 2 kB that the virus can package. Because of the present invention, more than 36 kB of heterologous DNA can be contained in the vector, depending on the size of the deletion. Different vectors lacking 10, 15, 20, 30 and 35 kB of adenoviral sequences are contemplated. The present invention makes possible, for example, deletion of the E1, E2, E3, E4, L1, L2, L3, L4 and L5 regions, or any combination of these regions, and replacement of the deleted regions with heterologous DNA.

The adenovirus vector construct must therefore replicate in a helper cell with the aid of one or more

helper viruses. In order for replication to occur, the vector must encode all of the necessary *cis*-acting elements needed for replication of the vector DNA, including those required for initiation of genome replication and for packaging of the replicated DNA into the viral capsid, provided that the remaining *trans* elements are supplied by the helper cell and the helper virus.

In the context of the adenovirus vector, the term heterologous DNA is meant to include DNA derived from a source other than the adenovirus genome which provides the backbone of the vector. This heterologous DNA may be derived from a prokaryotic or a eukaryotic source such as a bacterium, a virus, a yeast, a plant or even an animal. The heterologous DNA may also be derived from more than one source. For instance, a regulatory sequence may be derived from a virus and may control the expression of a structural gene from a different source, such as a mammal.

Regulatory elements include promoters. Preferred promoters are viral promoters such as the adenovirus major later promoter, SV40 late promoter from simian virus 40, the Baculovirus polyhedron enhancer/promoter element, Herpes Simplex Virus thymidine kinase (HSV *tk*), the immediate early promoter from cytomegalovirus (CMV) and various retroviral promoters including LTR elements. The elements are operably linked to a gene, the expression of which is desired. By "operably linked," it is meant that the regulatory element is positioned, relative to a coding sequence, such that expression of that coding sequences is effected or enhanced by that element.

The promoters and enhancers preferably employed will be those that control the transcription of protein

**F. Helper Virus**

Another aspect of the present invention is the helper virus. A helper virus is defined as an adenovirus that can complement the replication and packaging of the adenovirus vector construct. Helper vectors require the same *cis*-acting sequences as the adenoviral vectors, namely, an origin of replication and a packaging signal. Preferably, a helper virus according to the present invention also contains a mutation in the adenovirus packaging signal that causes it to be utilized less efficiently than the wild-type packaging signal, although it still is utilized to an extent that packaging will occur in the absence of competing, wild-type signals. Like the adenoviral vectors, the helper virus will need to be propagated on a helper cell line that compensates for its defects. Usually, the defects will include deletions in the E1 and/or E2 regions of the helper virus genome. Also, the non-essential E3 region may be removed. A list of some possible combinations is provided in the following table.

**TABLE 1: PHENOTYPES OF VARIOUS COMPONENTS**

| VECTOR | HELPER CELL | HELPER VIRUS |
|---|---|---|
| E4 | E1 | E2, L1-L5 |
| E4 | - | E1, E2, L1-L5 |
| - | E1 | E2, E4, L1-L5 |
| - | E1, E4 | E2, L1-L5 |
| - | E4 | E1, E2, L1-L5 |

\- signifies absence of functional E1-E5 and L1-L5 products

One of the advantages provided by the present system is the greatly reduced possibility that the adenoviral vector, through homologous recombination, will reacquire

A first step in expressing a foreign gene is the development of adenoviral helper viruses. The general scheme for generating helper viruses is as follows. First, a part of the adenoviral genome is inserted into a standard cloning vector. A deletion in that region is then engineered. Following amplification of the deletion construct, the adenoviral fragment is excised and cotransfected with adenoviral genomic DNA in a cell line expressing the "early" functions deleted from the adenoviral fragment. Following recombination, adenoviral genomic DNA lacking the deleted sequences are isolated. This process can be repeated to incorporate additional deletions so long as cell lines are available that can complement the increasing number of defective functions.

In a preferred embodiment of the foregoing, the adenoviral genomic DNA that receives the deletion fragments contains a mutation in the packaging signal ($pac^-$). Because the recombination of deletions into the adenoviral genome is accomplished in the absence of other adenoviruses, however, the mutated packaging signal is sufficient to permit encapsidation. Subsequent propagation of this virus with vectors containing wild-type packaging signals will result in preferential encapsidation of the vectors.

Also important is the development of Ad helper cell lines. These cells lines are the stably transfected, or "transformed," with DNA from adenovirus. The DNA may be integrated or maintained as episomal fragments of Ad sequences. These cell lines are designed to express different sets of Ad proteins and can be used to generate and propagate different Ad vectors.

Another step in the helper virus system is the construction of adenoviral vectors. Ad vectors can be generated in two basic fashions. First, a region of the

adenoviral genome can be inserted into a standard cloning vector. Next, a deletion is engineered into the adenovirus insert and the deleted sequences replaced with a heterologous DNA. Cotransfection of the Ad-hetDNA-Ad insert with adenoviral genomic DNA will result in recombination of the heterologous DNA, by virtue of the flanking Ad sequences, into the cotransfected adenoviral genomic DNA. If the host cells do not compensate for the adenoviral functions missing from the new recombinant, the cells can be superinfected with helper viruses.

An alternative method for generating Ad vectors would be the generation, *in vitro*, of the entire Ad sequences. Using restriction enzymes and DNA ligase, it is possible to clone directly the necessary *cis*-acting sequences from adenovirus to the heterologous DNA. This construct can then be transfected into helper cells, optionally infected with helper virus, for the purposes of replication and encapsidation. In this context, it may be helpful to generate an adenoviral vector containing only the packaging signal, origin of replication and a multipurpose cloning site for the insertion of heterologous DNA. In a preferred embodiment, this starting vector also would contain an excisable marker gene.

In any of the preceding or following discussion, the term transfecting should be understood as including an type of gene transfer methodology including calcium-phosphate precipitation, protoplast fusion, lipofection, cation-facilitated DNA (*e.g.*, polylysine) transduction or any other equivalent method. Together, these terms are deemed equivalent the phrase "importing nucleic acid."

For example, technology is available to conjugate naked nucleic acids to polycations, which conjugates are taken up by cells brought in contact with such

conjugates. In certain embodiments, it also is desirable to include an agent capable of disrupting lysosomes in which the conjugate is taken up. A preferred lysosomal disruption agent is adenovirus itself. The adenovirus can be wild-type adenovirus, adenovirus containing a defective genome or empty adenovirus particles. An example of the approach is illustrated in FIG. 7.

## EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1: Construction of a Packaging Defective Adeno-Helper Virus

A helper virus was generated by recombination of overlapping sequences of a shuttle vector and cloned fragments of the adenovirus genome. The *EcoRI-ClaI* small fragment from pXCJ.2 (Spessot *et al.*, 1989) was subcloned into the respective sites of pBSKS (Stratagene) to generate pBSleft-end. A 1.7 kB fragment from nucleotides 4021 to 5785 of Ad5 was synthesized by PCR using pJM17 (McGrory *et al.*, 1988) as a template using primers 5'-CCATCGATGCGGTTTAAAACATAAAT-3' (*ClaI* site underlined) and 5'-CCGCGGAACACCGCTCGAGGAC-3'. pRA was generated by

inserting the PCR-generated fragments into *ClaI-XhoI* sites of pBSleft-end. Subsequent cleavage of pRA with *SgraI* (nucleotide 188) and *ClaI* (nucleotide 450), effectively removing the wild-type packaging signal. The corresponding *SgraI-ClaI* fragment from pE1A-10/28, containing a double-deletion in the Ad5 packaging signal (Grable and Hearing, 1990) was inserted into pRA. pJM17 and pRApac$^-$ were cotransfected into 293 cells. Virus is then purified and cloned by limiting dilution on 293 cells, after which pac$^-$ helper is identified.

**Example 2: Construction of a Replication Deficient Adenovirus Vector**

By fusing the inverted terminal repeats (ITRs) of adenovirus with a prokaryotic origin of replication, a replicable adenovirus vector can be constructed that also may be propagated in bacteria. Such a vector has been generated by inserting the ITR fusion region of pAB17 (*BsaAI* site at 35,771 to *SacII* site at 358 (nucleotide nos. from Ad5) into the *XbaI* site of pREP9 (Invitrogen). This insert contains the wild-type packaging signal at nucleotides 194-358. As a reporter, the ß-gal gene was subcloned as a *NotI-NotI* fragment from pTKß (Clontech) into the *NotI* site following the Rous Sarcoma Virus promoter of pREP9.

**Example 3: Expression of Heterologous Peptide in Mammalian Cells**

p53 is excised from pEC53 (Zhang *et al.* 1994) and inserted into the *NotI* site of the vector described in example. The plasmid is introduced into 293 cells by Dotap-mediated transfection. *Id.* After 24 hours, cells are infected with the helper virus described in Example 1 in complete medium. After cytopathic effects appear, virus is harvested by three freeze-thaw cycles and virus

purified by CsCl density gradients. *Id.* Purified vector is stored at -80°C in buffered 10% glycerol.

**Example 4: Administration of a Therapeutic Vector to a Patient *In Vivo***

Large scale production of viral vector described in Example 3 will be undertaken and each batch evaluated for purity and homogeneity. Virus stocks can be stored at titers of $10^{11}$ pfu/ml at -80°C. Patients with advanced (stage III) inoperable adenocarcinoma are selected for possible treatment and the tumors screened for p53 status. Those patients having tumors with deleted or mutated p53 are further selected for treatment. By fibroscopy, as much tumor mass as possible is removed and a fibroscopic-guided needle is used to inject vector at 0.1 ml volumes ($10^{10}$ pfu) at 4-6 sites in the remaining tumor mass. Patients are monitored daily for systemic inflammation. After about 1 week, the tumor is biopsied to assess p53 expression and vector presence. Pharyngeal mucosa, urine and stool samples are taken in order to assess for possible adventious virus shedding.

6. The vector of claim 1, wherein said vector lacks the coding regions for adenoviral products E1A, E1B, E2, E3 and L1-5.

7. The vector of claim 1, wherein said vector lacks the coding regions for adenoviral products E1A, E1B, E2, E3, E4, L1, L2, L3, L4 and L5.

8. The vector of claim 7, wherein vector lacks all adenoviral coding regions.

9. An isolated adenovirus vector, wherein said vector comprises an adenoviral inverted terminal repeat and an adenoviral packaging signal and a non-functional, non-immunogenic form of

(i) the adenoviral products E1A, E1B and E3; and

(ii) at least one of the adenoviral products selected from the group consisting of E2, E4, L1, L2, L3, L4 and L5.

10. The vector of claim 1, wherein said vector further comprises a heterologous DNA of at least 10 kB.

11. The vector of claim 1, wherein said vector further comprises a heterologous DNA of at least 15 kB.

12. The vector of claim 11, wherein said vector further comprises a heterologous DNA of at least 20 kB.

13. The vector of claim 12, wherein said vector further comprises a heterologous DNA of at least 30 kB.

14. The vector of claim 13, wherein said vector further comprises a heterologous DNA of about 35 kB.

15. The vector of claim 10 further comprising a promoter, wherein said heterologous DNA is operably linked to said promoter.

16. The vector of claim 1, wherein said heterologous DNA encodes a tumor suppressor.

17. The vector of claim 1, wherein said heterologous DNA encodes a product involved with cystic fibrosis.

18. The vector of claim 1, wherein said heterologous DNA encodes a product involved with Duchenne muscular dystrophy.

19. The vector of claim 1, wherein said heterologous DNA encodes an antisense construct.

20. The vector of claim 15, wherein said promoter is an adenoviral major late promoter.

21. The vector of claim 15, wherein said promoter is a heterologous, cell-specific promoter.